Quantitative Analysis of Plasma TP53 249^{Ser}-Mutated DNA by Electrospray Ionization Mass Spectrometry

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Abstract

A mutation in codon 249 of the *TP53* gene (249^{Ser}), related to aflatoxin B_1 exposure, has previously been associated with hepatocellular carcinoma risk. Using a novel internal standard plasmid, plasma concentrations of 249^{Ser}-mutated DNA were quantified by electrospray ionization mass spectrometry in 89 hepatocellular carcinoma cases, 42 cirrhotic patients, and 131 nonliver diseased control subjects, all from highly aflatoxin-exposed regions of The Gambia. The hepatocellular carcinoma cases had higher median plasma concentrations of 249^{Ser} (2,800 copies/mL; interquartile range: 500-11,000) compared with either cirrhotic (500 copies/mL; interquartile range: 500-2,600) or control subjects (500 copies/mL; interquartile range: 500-2,000; $P < 0.05$). About half (52%) of the hepatocellular carcinoma cases had >2,500 copies of 249^{Ser}/mL plasma, corresponding to the prevalence of this mutation in liver tumors in The Gambia. In comparison, only 15% of control group and 26% of cirrhotic participants exceeded this level ($P < 0.05$). Further subset analysis revealed a statistically significant, quantitative relation between diagnosis of hepatocellular carcinoma and levels of 249^{Ser} detected at 2,501 to 10,000 copies/mL plasma (odds ratio, 3.8; 95% confidence interval, 1.3-10.9) and at >10,000 copies/mL plasma (odds ratio, 62; 95% confidence interval, 4.7-820) when compared with control subjects and after adjusting for age, gender, recruitment site, hepatitis B and C serologic status, and total DNA concentration. Levels of >10,000 copies of 249^{Ser}/mL plasma were also significantly associated with the diagnosis of hepatocellular carcinoma (odds ratio, 15; 95% confidence interval, 1.6-140) when compared with cirrhotic patients. Potential applications for the quantification of 249^{Ser} DNA in plasma include estimation of long-term, cumulative aflatoxin exposure and selection of appropriate high-risk individuals for targeted intervention. (Cancer Epidemiol Biomarkers Prev 2005;14(12):2956–62)

Introduction

Hepatocellular carcinoma is a major cause of cancer death in sub-Saharan Africa and Asia (1). The primary etiologic factors associated with development of hepatocellular carcinoma are chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), and, in many regions, exposure to aflatoxin B_1 in the diet (2). Tumor-specific TP53 mutations have been identified in several human cancers. A ''hotspot'' mutation identified in hepatocellular carcinoma is the selective guanine-to-thymine transversion mutation (249^{Ser}) in codon 249 (AGG to AGT; arginine-to-serine substitution) of the *TP53* gene (3). This mutation is often detected in hepatocellular carcinoma from populations exposed to aflatoxin B_1 and with a high prevalence of HBV carriers (4, 5). In The Gambia (West Africa), hepatocellular carcinoma is the most common cancer among men and second most common among women (6). A recent study in The Gambia identified the 249^{Ser} mutation in ~40% of tumors from hepatocellular carcinoma patients (7). Around 15% of Gambians have been chronically infected with HBV (8) and >95% of the population has had detectable levels of aflatoxin-albumin adducts in their serum (9).

Increasing focus and research on circulating cell-free DNA has shown that DNA can be isolated from the plasma or serum of most healthy individuals (10). Although the precise mechanism by which free DNA enters the circulation remains unclear, release of DNA after cell death (either due to necrosis or apoptosis), active release from cells, and cellular injury have been suggested as possible mechanisms (11). A number of recent studies have suggested that cancer patients have significantly higher levels of circulating DNA compared with healthy subjects (12-14) and that the level of circulating DNA in plasma may be useful as a diagnostic marker for some types of cancer (15, 16), including hepatocellular carcinoma (17).

The presence of plasma-derived DNA with the same genetic alterations present in the tumor should be a more informative and specific biomarker of a particular cancer than the level of circulating normal DNA. In an earlier study from The Gambia, the detection of TP53 249^{Ser} mutation in plasma DNA by restriction digestion methods (RFLP-PCR) was strongly associated with hepatocellular carcinoma (18). In two separate studies, one from China (19) and another from The Gambia (20), the presence of TP53 249^{Ser}-mutated DNA in plasma correlated strongly with the presence of the mutation in paired tumors from the same individuals. Short oligonucleotide mass analysis (SOMA; ref. 21), a method combining PCR amplification, restriction digestion, and electrospray ionization mass spectrometry, was shown to be more sensitive than RFLP-PCR for detecting the TP53 249^{Ser}-mutated DNA in plasma (22).

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In this study, we have developed a method to determine plasma concentrations of TP53 249^{Ser}-mutated DNA through incorporation of a novel internal standard plasmid step into the SOMA method. Using this approach, we evaluated the ability of TP53 249^{Ser}-mutated DNA levels in plasma to differentiate individuals with hepatocellular carcinoma from cirrhotic patients and nondiseased control subjects, all of whom were highly exposed to aflatoxin.

Materials and Methods

Study Population. The Gambia Liver Cancer Study recruited patients with hepatocellular carcinoma and cirrhosis from liver disease referral clinics and nonliver diseased control participants (Table 1) from general outpatient clinics at three tertiary hospitals in The Gambia (23). Incident hepatocellular carcinoma cases (n = 89) were defined by clinical history and ultrasonographic findings by physicians experienced in liver cancer diagnosis. Seventy-two percent of cases were additionally confirmed by elevated α-fetoprotein levels and 23% of the remaining hepatocellular carcinoma cases were confirmed by pathologic findings. Cirrhosis diagnosis (n = 42) was based on ultrasonographic findings without focal lesions suggestive of hepatocellular carcinoma. Nonliver diseased control participants (n = 131), without clinical evidence of liver disease, were frequency matched by age (within 10 year groupings), gender, and recruitment site. Evaluation of all participants included a detailed interviewer-administered questionnaire, a physical exam, collection of biological specimens, and a standardized ultrasound examination in the case of hepatocellular carcinoma and cirrhotic participants. All case-control study participants signed an informed consent and the study was approved by ethical review boards in The Gambia, at National Cancer Institute and at IARC.

Extraction, Purification, and Quantification of Plasma DNA. DNA was extracted from 200 μL of plasma, using the QIAamp DNA Blood Mini kit according to the blood and body fluid spin protocol provided by the manufacturer (Qiagen, Chatsworth, CA). Purified DNA was eluted from the QIAamp Silica column with two 50 μL volumes of nuclease-free water (PCR-grade, Sigma Chemical Company, St. Louis, MO). Plasma extract DNA concentrations were measured by fluorescence using a Picogreen double-stranded DNA quantitation kit (Invitrogen, Cergy Pointoise, France).

Detection of TP53 249^{Ser} Mutation by RFLP-PCR. The detection of TP53 249^{Ser}-mutated DNA in plasma by RFLP-PCR has been described previously (18). Briefly, 2 to 8 μL DNA extract was used to amplify a 237-base sequence flanking exon 7 of the *TP53* gene corresponding to Genbank nucleotides 13,941 to 14,177 (accession no. U94788) using 0.2 μmol/L (final concentration) of the following primers: 5′-CTTGCCACAG-GTCTCCCCAA-3′ and 5′-AGGGGTCAGCGGCAAGCAGA-3′

Table 1. Characteristics of the aflatoxin-exposed study population

	Nonliver diseased controls	Cirrhosis	Hepatocellular carcinoma
No. subjects	131	42	89
Mean age, y (range)	43 (19-80)	44 (21-71)	50 (20-85)
No. males (%)	93 (71)	24 (57)	71 (80)
No. HBsAg+ subjects* (%)	20 (16)	23 (55)	51 (58)
No. anti-HCV+ subjects* (%)	2 (2)	1 (3)	14 (17)

Abbreviation: HBsAg, HBV surface antigen.
*A few participants had missing data for HBsAg (one hepatocellular carcinoma case and three controls) and for anti-HCV (six hepatocellular carcinomas, three cirrhosis, and four controls) data.

(Proligo, Paris, France). When necessary, a second PCR reaction was used to amplify a nested 177-base sequence corresponding to Genbank nucleotides 13,960 to 14,136, using the following primers: 5′-AGGCGCACTGGCCTCATCTT-3′ and 5′-TGTGCAGGGTGGCAAGTGGC-3′ (Proligo). PCR product (10 μL) was digested by *Hae*III restriction endonuclease (Roche Diagnostics France, Meylan, France), which cuts within a GG|CC sequence encompassing codons 249 and 250 (AGGCCC; Genbank nucleotides 14,072-14,077). Digestion of wild-type (WT) DNA generates two bands of 92 and 66 bp, whereas mutant material, in which the restriction site has been destroyed, yields a noncleaved band of 158 bp. Mutant fragments, visualized on 3% agarose gel stained with ethidium bromide, were cut out of the gel, reamplified, purified with a QIAquick PCR Purification kit (Qiagen), and sequenced by automated, dideoxy sequencing (Prism 3100 Genetic Analyser, Applied Biosystems, Foster City, CA). All analyses were repeated at least twice.

Quantification of 249^{Ser} Mutated DNA in Plasma Extracts by SOMA

Design of the Mutant TP53 Internal Standard Plasmid. In a previous study, to generate knock-in mice with a chimeric human/murine *TP53* gene (24), 86 bp (42,025 Da) of a pBluescript SK (−) plasmid (1,828,595 Da; Stratagene, La Jolla, CA) were replaced with a 5,765 bp segment of the human *TP53* gene delimited by the end of intron 3 and the beginning of intron 10 (3,506,585 Da). This plasmid (5,294,155 Da) was used as a template for preparation of the internal standard plasmid for this study. Using directed mutagenesis (TOPO-2; Invitrogen), a G-to-T point mutation was introduced into the plasmid at the third base of TP53 codon 249 using the following primers: (TP53-^{249}SER-sense) 5′-GGCATGAACCGGAG*T*CC-CCATCCTC-3′ and (TP53-^{249}SER-antisense) 5′-GAGGATGG-GACTCCGGTTCATGCC-3′. Using the same procedure, a G-to-C mutation was then introduced into this modified plasmid at the third base of TP53 codon 248 using the following primers: (TP53-IS-sense) 5′-GGCATGAACCG*G*AG*T*CC-CCCATCCTC-3′ and (TP53-IS-antisense) 5′-GAGGATGG-GACTGCGGTTCATGCC-3′. Thermal cycling conditions for both PCR amplifications were 95°C for 3 minutes, followed by 12 cycles of 95°C for 30 seconds, 55°C for 60 seconds, and 68°C for 17 seconds, followed by a final time of 5 minutes at 72°C. Following bacterial transformation (DH5α, Invitrogen) of the mutant plasmid, 12 colonies were randomly picked: six colonies (1A1, 1A2, 1B1, 1B2, 1C1, and 1C2) were shown by both SOMA and sequencing to contain both mutations and six colonies (3A1, 3A2, 3B1, 3B2, 3C1, and 3C2) had only the single mutation at codon 249. Further studies were carried out with plasmid clones 1A1 and 3A1.

SOMA PCR Amplification of DNA Extracted from Plasma. Three hundred thirty-three copies of internal standard plasmid 1A1 and 1 to 10 μL of plasma extract, containing from 0.1 to 3 ng of genomic DNA, were amplified by two consecutive rounds of 38 PCR cycles in a 25 μL total volume containing 2.5 μL of 200 mmol/L Tris-HCl (pH 4.8), 0.6 μL of 50 mmol/L $MgCl_2$, 3 μL containing 1.7 mmol/L each of the four deoxynucleotide triphosphates, 0.3 μL of 5 units/μL Taq-Platinum (Invitrogen), 0.5 μL of forward and reverse primers at 350 ng/μL, and 12.6 μL water, or, for the second PCR amplification, 12.6 μL water and 8 μL of PCR1 amplification product. Thermal cycling conditions for both PCR amplifications were 95°C for 2 minutes, followed by 38 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. The 93 bp TP53 sequence used for SOMA analysis corresponds to Genbank nucleotides 14,026-14,118 (accession no. U94788) using the following SOMA PCR primers: 5′-GGTGTTTGT-GGGGAGGGGGTTCTGGAGTTTAGGAAGTATAGTT-3′ and 5′-AACCACTAAACACACAACTCTACTGGAGAAAACCA-ATAAAAA-3′. The six-base recognition sequence CTGGAG for the type II restriction enzyme *Gsu*I (Fermentas, Vilnius, Lithuania) was incorporated into the forward primer at nucleotides 14,047 to 14,052 and into the reverse primer at nucleotides 14,091 to 14,096.

Restriction Digestion of SOMA Oligonucleotides. Restriction digestion of 20 μL of this amplified DNA mixture was carried out overnight at 30°C in a final volume of 50 μL containing 2 μL of 1 unit/μL *Gsu*I; 5 μL of a mixture of 10 mmol/L Tris-HCl (pH 7.5), 10 mmol/L $MgCl_2$, and 0.1 mg/mL bovine serum albumin; and 23 μL of water (PCR grade, Sigma). This procedure produces 8-mer DNA fragments (Fig. 1) containing both codons 248 and 249. Amplification and restriction digestion are independent of the 8-base sequence so a mixture of internal standard (249^{IS}), WT (249^{WT}), and 249^{Ser}-mutated DNA fragments are produced.

Purification of SOMA Oligonucleotides. The digestion products (50 μL) were then mixed with 100 μL of phenol/chloroform/isoamyl alcohol (25:24:1, v/v; Invitrogen) and 50 μL of water (molecular biology grade, Eppendorf, Hamburg, Germany). After centrifugation for 5 minutes at 10,500 × *g* at room temperature, the aqueous upper layer was removed to another tube. DNA was coprecipitated with 2 μL See-DNA (Amersham, Orsay, France) after addition of 30 μL of 7.5 mol/L ammonium acetate (Sigma), to reduce the level of sodium adduct ions, and 500 μL of ethanol, with storage at −80°C for at least 30 minutes. After another centrifugation at 10,500 × *g* at 4°C for 10 minutes, the precipitated pink DNA pellet was washed with 500 μL cold 70% ethanol and again centrifuged at 10,500 × *g* at 4°C for 15 minutes. Before high-performance liquid chromatography (HPLC)–tandem mass spectrometry analysis, the air-dried DNA pellets were resuspended with mixing in 6 μL of HPLC mobile phase, a solution of aqueous 0.4 mol/L 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma) and methanol (80:20, v/v).

HPLC Conditions. Further HPLC (CapLC, Waters-Micromass, Manchester, United Kingdom) purification was carried out at 25 μL/min on a 15 cm × 800 μm I.D Vydac C-18 reversed phase column (5 μm, 300 Å pore size; LC Packings, Amsterdam, the Netherlands). HPLC solvents were prepared from a stock solution of aqueous 0.8 mol/L 1,1,1,3,3,3-hexafluoro-2-propanol, adjusted to pH 7.0 with triethylamine, then diluted to 0.4 mol/L (with water for solvent A and methanol for solvent B). An initial mobile phase concentration of 20% B was programmed to 100% B in 7.1 minutes. The entire 6 μL sample was injected onto the HPLC column, as under these initial HPLC conditions, oligonucleotides are concentrated at the head of the column and low molecular weight salts and other impurities are washed away. SOMA oligonucleotides elute at ∼5 minutes.

Mass Spectrometric Analysis of SOMA Oligonucleotides. As the DNA fragments elute into the mass spectrometer, they dissociate into pairs of sense and antisense 8-mer oligonucleotides, which contain the internal standard, WT, or mutated sequence (Fig. 1). Electrospray ionization mass spectrometry of such 8-mer oligonucleotides produces a series of multiply charged ions. However, using the HPLC mobile phase described above, the negative electrospray mass spectrum for the WT sense TP53 oligonucleotide (Fig. 2A) shows a major peak for the $[M\text{-}2H]^{2-}$ ion at *m/z* 1,256.7. Addition of 1,1,1,3,3,3-hexafluoro-2-propanol to the HPLC mobile phase increases the relative abundance of doubly charged ions, minimizing sodium ion adduction (Fig. 2A, *inset*) and maximizing the sensitivity of the method.

To obtain sequence information, the first sector of the tandem mass spectrometer is set to selectively pass ions with a mass-to-charge ratio corresponding to the $[M\text{-}2H]^{2-}$ ion of the selected oligonucleotide. This beam of ions is then collided, at an energy of 37 V, with a curtain of argon gas to produce a spectrum of sequence-specific fragment ions (Fig. 2B), which are analyzed by the second sector of the tandem mass spectrometer. An a_x-B_x series of fragment ions is formed from the 5′ end of the oligonucleotide and a w_x series of fragment ions from the 3′ end. Fragment ions specific for 249^{WT}, 249^{Ser}, and 249^{IS} oligonucleotides are monitored as the compounds elute into the mass spectrometer. For example, Fig. 2A shows the full-scan mass spectrum for the 249^{WT}-sense oligonucleotide and Fig. 2B the full-scan daughter ion spectrum this parent ion. Thus, for specific detection of G-to-T variant sequences in TP53 codon 249, the mass spectrometer was programmed to acquire data in the selected reaction monitoring mode by monitoring six sequence-specific $[M\text{-}2H]^{2-}$ ion fragments: (249^{WT}-sense): 1,256.8→924.6; (249^{WT}-antisense): 1,219.8→1,059.6; (249^{Ser}-sense): 1,244.3→899.6; (249^{Ser}-antisense): 1,231.8→1,083.7; (249^{IS}-sense): 1,224.3→889.6; and (249^{IS}-antisense): 1,251.8→1,083.7. Before integration of 249^{WT}, 249^{Ser}, or 249^{IS} peak areas, signals for sense and antisense oligonucleotides were summed.

Selected reaction monitoring mass chromatograms were obtained on a LC Quattro mass spectrometer (Waters-Micromass) equipped with an electrospray ionization source operated in the negative ionization mode. The electrospray capillary was held at −5 kV and the cone potential was typically 77 V. Ion source temperature was 200°C and the electrospray desolvation temperature was 215°C. Argon pressure in the collision cell was $\sim 5 \times 10^{-3}$ mbar.

Quantification. The six synthetic 8-mer oligonucleotides (Proligo) shown in Fig. 1 were diluted with HPLC mobile phase to ∼10 ng/μL, checked for purity by HPLC mass spectrometry, and carefully quantified by UV. Seven calibration

	SOMA 8-mer DNA fragments	SOMA 8-mer oligonucleotides	CID fragment ion	
249^{WT}-s	pCGG AG**G** CC	m/z 1256.8 pCGG AG**G** CC	m/z 924.6 **G** CC	w_3^-
249^{WT}-as	TG GCC TC**C**p	m/z 1219.8 p**C**CT CCG GT	m/z 1059.6 p**C**CT..	$[a_4\text{-}B_4]^-$
249^{Ser}-s	pCGG AG**T** CC	m/z 1244.3 pCGG AG**T** CC	m/z 899.6 **T** CC	w_3^-
249^{Ser}-as	TG GCC TC**A**p	m/z 1231.8 p**A**CT CCG GT	m/z 1083.7 p**A**CT..	$[a_4\text{-}B_4]^-$
249^{IS}-s	pCG**C** AG**T** CC	m/z 1224.3 pCG**C** AG**T** CC	m/z 899.6 **T** CC	w_3^-
249^{IS}-as	TG GC**G** TC**A**p	m/z 1251.8 p**A**CT **G**CG GT	m/z 1083.7 p**A**CT..	$[a_4\text{-}B_4]^-$

Figure 1. Base sequences and tandem mass spectrometry transitions for 249^{WT}, 249^{Ser}, and 249^{IS} SOMA oligonucleotides.

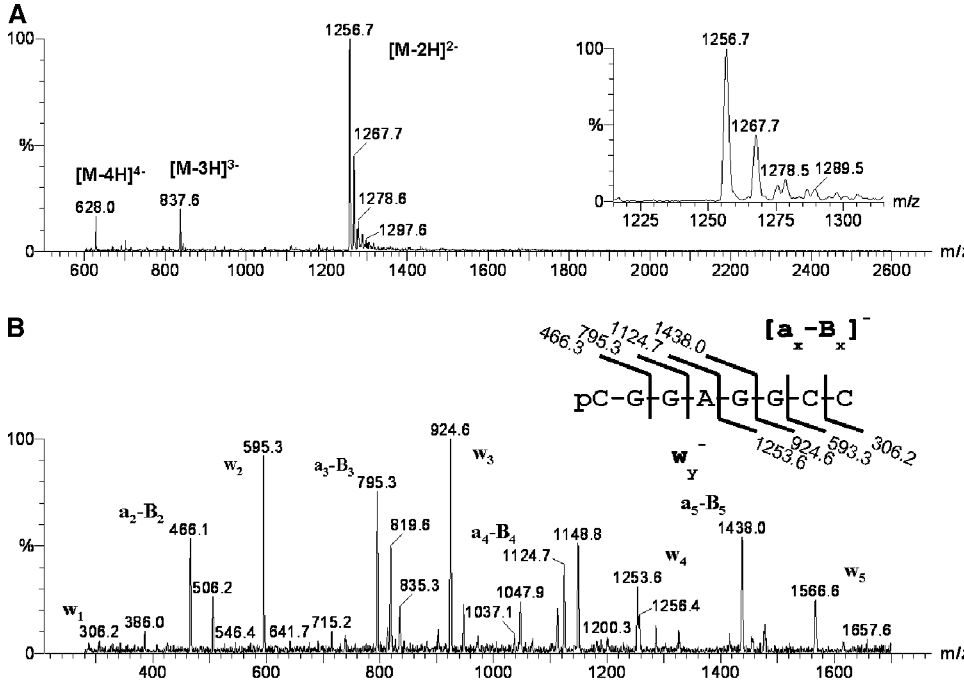

Figure 2. A. Negative electrospray mass spectrum of 249^{WT} sense oligonucleotide. **B.** CID mass spectrum of the $[M\text{-}2H]^{2-}$ oligonucleotide ion (*m/z* 1,256.8).

solutions were prepared, containing the six oligonucleotides (both sense and antisense) in the following ratios (w/w/w): ($249^{IS}/249^{WT}/249^{SER}$, 333:0:0, 333:17:17, 333:33:33, 333:84:84, 333:167:167, 333:333:333, and 333:666:666). These solutions, injected with each batch of human samples, were used to prepare the calibration curves. Coefficients of determination for both curves, fit with a first-order quadratic equation, were >0.999. The limit of detection for both 249^{WT} and 249^{Ser} DNA, relative to 333 copies of 249^{IS} plasmid DNA, was 10 copies. Thus, depending on whether 20, 10, 5, or 2.5 µL of plasma was used for the analysis, the limit of determination for the method was 500, 1,000, 2,000, or 4,000 copies DNA/mL plasma, respectively. For statistical analysis, samples below the detection limit were assigned a value of one-half the limit of determination. Mass spectrometry–selected reaction monitoring results for typical human samples are shown in Fig. 3.

To measure the reproducibility of the quantitative SOMA method, four aliquots of a plasma sample were analyzed in parallel. Average 249^{Ser} levels were 9,510 ± 990 copies/mL (coefficient of variation = 10%) and average 249^{WT} levels were 126,880 ± 17,460 copies/mL (coefficient of variation = 14%).

Plasma DNA concentration, measured by SOMA, was correlated with the plasma DNA concentration determined by fluorescence for the 262 subjects. Correlation of results determined by the two methods was good ($r^2 = 0.67$) with the SOMA method finding ~81% of the value determined by fluorescence (correlation equation: $y = 0.81 + 1{,}900$). This implies that the quantitative SOMA method described here provides measurements that are of comparable reliability to the fluorescence method using Picogreen.

Statistical Analysis

Analysis of 249^{Ser} Plasma DNA Levels and Disease Outcomes. Our primary evaluation was to examine plasma 249^{Ser} levels in relation to risk for hepatocellular carcinoma compared with nonliver diseased control subjects. We also investigated the association of plasma 249^{Ser} levels in relation to cirrhosis compared with nonliver diseased controls and, additionally,

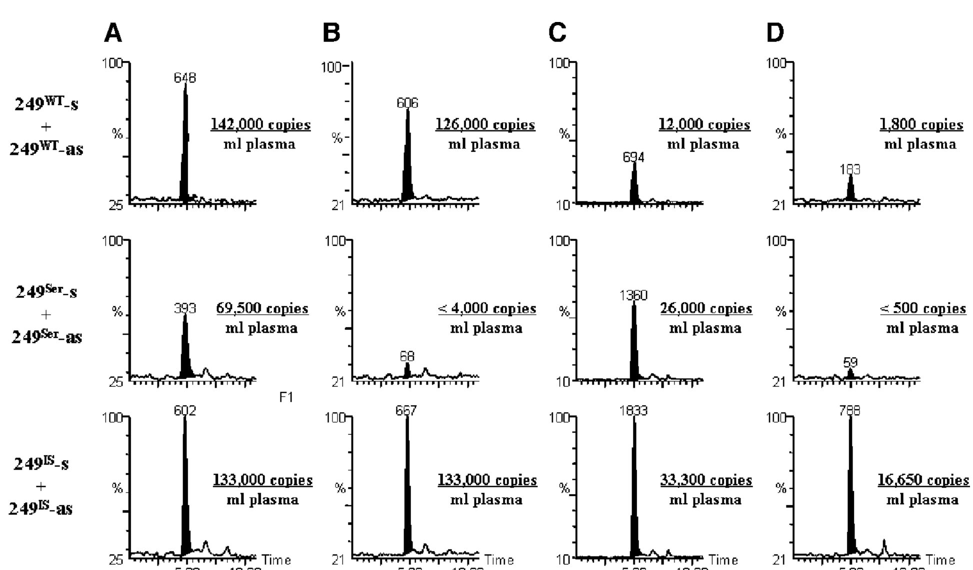

Figure 3. Typical selected reaction monitoring chromatograms for SOMA PCR amplifications involving 333 copies of internal standard and 2.5 µL (**A** and **B**), 10 µL (**C**), and 20 µL (**D**) of plasma. For each sample, the mass spectrometer was set to scan transitions specific for the 249^{WT}, 249^{Ser}, and 249^{IS} sense and antisense oligonucleotides. *Numbers above peaks,* peak areas.

of hepatocellular carcinoma cases compared with cirrhotic patients. These dichotomous disease outcome variables (hepatocellular carcinoma cases versus control, cirrhosis versus control, and hepatocellular carcinoma cases versus cirrhosis) were sequentially analyzed by similar methods. Association of outcome variables with predictor variables were evaluated for statistical significance by Pearson's χ^2 and Fisher's exact tests. All P values reported are two-tailed. In multivariable analysis, odds ratios (OR) with 95% confidence intervals (95% CI) were estimated as measures of association by stepwise unconditional logistic regression. Plasma 249^{Ser} levels (in copies/mL plasma) were categorized into four groups based on their distribution within the data set (<500; 500-2,499; 2,500-9,999; and ≥10,000). Although the distribution of 249^{Ser} levels by study group is presented for each category, the lowest two categories were collapsed into a single reference group for the risk estimate analysis. The final multivariable model presented included adjustment for variables known to be associated with hepatocellular carcinoma (including age, gender, and HBV and HCV status) and also variables related to implementation of the study (age, gender, and recruitment site) or to detection of plasma 249^{Ser} (age and total free DNA concentration). Comparative risk estimates between plasma 249^{Ser} levels (dichotomized as <2,500 or ≥2,500 copies/mL) and the dichotomous 249^{Ser} status as determined by RFLP methods were done using the identical analytic model and participants. Analyses were done using Stata statistical software (College Station, TX).

Results

Greater than 95% of all plasma samples from The Gambia that have been analyzed have been found to contain aflatoxin (25, 26). Thus, the demographic characteristics and viral serology results of the participants presented in Table 1 should be framed within this exposure context. The mean age of hepatocellular carcinoma cases was 50 years, which was ~6 to 7 years older than the cirrhotic and nonliver diseased control participants. The expected male predominance among hepatocellular carcinoma cases was observed, with a gender ratio of four males for every female. The majority of both hepatocellular carcinoma and cirrhotic subjects were chronically infected with HBV, as determined by antibodies to HBV surface antigen. HBV surface antigen prevalence among controls was 16%, consistent with the population prevalence among adult Gambians. Antibodies to HCV were uncommon among the control and cirrhotic participants (2-3%) but were found in 17% of hepatocellular carcinoma cases. Both HBV and HCV serologic markers were independently associated with hepatocellular carcinoma in crude and adjusted analyses (data not shown; ref. 7).

Exploratory Analysis of Determinants of 249^{Ser} Plasma DNA Levels. As the initial step in evaluation of a novel quantitative biomarker, it is important to identify correlates that may affect detection or quantification of the marker. We extensively evaluated a variety of epidemiologic (age, gender, geography, and season) and laboratory variables (DNA concentration, HBV and HCV markers, liver enzyme levels, and aspartate aminotransferase levels) for any effect these may have on our estimates of 249^{Ser} plasma DNA levels. Using linear regression methods with log-transformed 249^{Ser} levels as the continuous outcome variable, we found that study group, season, elevated aspartate aminotransferase levels, and DNA concentration were the primary determinants of 249^{Ser} concentration. Controlling for study group, threshold values of the highest quartile of total DNA concentration and the months of November to August were associated with significantly higher 249^{Ser} copies/mL plasma ($P < 0.05$ for both). Although age was initially associated with plasma 249^{Ser} levels, this was confounded by younger participants having higher DNA concentrations. Of the laboratory variables evaluated, evidence of hepatocyte damage (aspartate aminotransferase levels more than twice the normal) were significantly associated with higher 249^{Ser} levels ($P = 0.04$), whereas serologic markers of chronic viral hepatitis were not. Similar trends were observed when 249^{Ser} levels were examined as a categorical rather than a continuous variable.

Plasma Levels of DNA. Plasma levels of circulating, cell-free total DNA, WT DNA, and 249^{Ser}-mutated DNA, all measured by SOMA, are presented by study group in Table 2. The median levels of total DNA were sequentially higher in the control, cirrhotic, and hepatocellular carcinoma study groups ($P_{trend} < 0.05$). Similarly, levels of WT DNA displayed an increasing trend by study group ($P_{trend} < 0.05$). Whereas both cirrhotic and control groups had median 249^{Ser} levels around the limit of determination (500 copies/mL plasma for both), hepatocellular carcinoma cases had a median of 2,800 copies of mutated 249^{Ser}/mL plasma. The median fraction of free-circulating plasma DNA that was mutated among hepatocellular carcinoma cases was ~11%; <3% was mutated in the plasma of cirrhotic patients and controls.

249^{Ser} Levels and Hepatocellular Carcinoma Risk. Two thirds (67%) of cases had detectable plasma levels of 249^{Ser}. Whereas 85% of controls and 74% of cirrhotic patients had plasma 249^{Ser} levels below 2,500 copies/mL, only 48% of hepatocellular carcinoma cases were below this cut point (Table 3). Cirrhotics had almost as high a proportion with plasma 249^{Ser} levels from 2,501 to 10,000 as hepatocellular carcinoma cases (24% and 26%, respectively), both much greater than controls (13%). Of participants with the highest levels of plasma 249^{Ser}, almost all were hepatocellular carcinoma cases (23 of 27, 85%). A concentration-dependent increase in the observed hepatocellular carcinoma risk was seen with increasing 249^{Ser} levels (Table 3). The crude odds for hepatocellular carcinoma associated with plasma 249^{Ser} levels of 2,500 to 9,999 and for ≥10,000 copies/mL were 3.5 (95% CI, 1.7-7.2) and 20 (95% CI, 5.6-69), respectively. When these associations were adjusted for factors known to be associated with hepatocellular carcinoma or the study design, the same trend was observed (Table 3) but the point estimate for the highest category was notably increased (OR, 62; 95% CI, 4.7-820).

Table 2. Plasma concentrations of total DNA, WT DNA, and 249^{Ser}-mutated DNA in the three aflatoxin-exposed groups studied

	Nonliver diseased controls	Cirrhosis	Hepatocellular carcinoma
Total plasma DNA level			
Median copies/mL plasma (IQR)	16,000 (8,900-31,000)	24,000 (12,000-39,000)	30,000 (16,000-39,000)
WT plasma DNA level			
Median copies/mL plasma (IQR)	15,000 (8,700-27,000)	21,000 (10,000-33,000)	25,000 (14,000-38,000)
249^{Ser} plasma DNA level			
Median copies/mL plasma (IQR)	500 (250-2,000)	500 (500-2,600)	2,800 (500-11,000)

Table 3. Prevalence and adjusted risk for hepatocellular carcinoma cases compared with nonliver diseased controls or cirrhotic patients as a function of TP53 249^{Ser} plasma concentration

	Concentration of TP53 249^{Ser} DNA in copies/mL plasma			
	≤500	501-2,500	2,501-10,000	>10,000
No. controls (%)	71 (54)	40 (31)	17 (13)	3 (2)
No. cirrhotic cases (%)	22 (52)	9 (21)	10 (24)	1 (2)
Crude OR (95% CI)	1.0		1.9 (0.8-4.7)	2.4 (0.4-15)
Adjusted OR (95% CI)	1.0		1.9 (0.6-5.7)	6.3 (0.2-270)
No. hepatocellular carcinoma cases (%)	29 (33)	14 (16)	23 (26)	23 (26)
Crude OR (95% CI)	1.0		3.5 (1.7-7.2)	20 (5.6-69)
Adjusted OR (95% CI)	1.0		3.8 (1.3-10.9)	62 (4.7-820)
Adjusted OR (95% CI)	1.0 (versus cirrhosis as reference group)		1.8 (0.6-5.3)	15 (1.6-140)

A similar but much weaker pattern of increasing risk with higher plasma 249^{Ser} levels was observed for cirrhosis compared with controls but these risk estimates were not statistically significant (Table 3). With cirrhosis as the comparison group, there was a 15-fold increased risk (95% CI, 1.6-140) of hepatocellular carcinoma for subjects in the highest 249^{Ser} category. No significant increase in hepatocellular carcinoma risk compared with cirrhotics was observed with 2,500 to 9,999 copies of 249^{Ser}/mL plasma (Table 3).

Quantitative SOMA Compared to RFLP. Among the hepatocellular carcinoma cases compared with control subjects, the dichotomous outcome variable of 249^{Ser} mutation detection by RFLP methods was associated with a 12-fold significant increase in hepatocellular carcinoma risk in adjusted analysis compared with controls (95% CI, 2.3-17). This risk estimate is between that observed for the two higher categories of plasma 249^{Ser} levels as detected through quantitative SOMA (Table 3). The two methods generally classified participants similarly. In evaluation of the agreement between the methods, we found an overall 74% agreement between the RFLP binary outcome and plasma 249^{Ser} levels dichotomized as above or below 2,500 copies/mL. In evaluation of both methods for discriminating between hepatocellular carcinoma cases versus controls, the quantitative levels provided a marginally improved area under the curve in receiver operating characteristic analysis compared with the RFLP method (0.717 versus 0.670). However, the two methods seem to provide complementary data; a combined variable that incorporated RFLP status and plasma 249^{Ser} levels (as classified in Table 3) provided the best predictive ability for hepatocellular carcinoma (0.739). Besides distinguishing between study groups, the quantification of 249^{Ser} levels also increased the overall prevalence of detection of 249^{Ser} circulating in plasma within each study group. The SOMA method identified 16%, 26%, and 52% of control, cirrhotic, and hepatocellular carcinoma patients, respectively, as having at least 2,500 copies of 249^{Ser}/mL plasma. This is compared with 5%, 14%, and 38% of these respective groups with detection by RFLP methods. All plasma DNA samples were also confirmed by DNA sequencing, a technique that is somewhat less sensitive in detecting this mutation than either RFLP-PCR or SOMA. Among the 25 of 89 cases found positive for the 249^{Ser} mutation by both RFLP-PCR and SOMA, 18 of 25 were confirmed positive by sequencing. Among the 21 of 89 hepatocellular carcinoma cases found positive only by SOMA, 12 of 21 were confirmed positive by sequencing. Among the 10 of 89 hepatocellular carcinoma cases found positive by only by RFLP-PCR, none were confirmed positive by sequencing.

Discussion

We report a novel application of the SOMA method for the quantification of a somatic mutation in free-circulating DNA in human plasma. The mutation we have chosen to study, a G-to-T (Arg to Ser) transversion at the third base of codon 249 in the *TP53* gene, has been observed with high prevalence and in strong association with the occurrence of hepatocellular carcinoma in regions of the world where aflatoxin B_1 exposure is high. Experimental evidence to support the carcinogen-specific nature of this mutation includes the selective formation of the major aflatoxin B_1-N^7-guanine adduct at this site and the predominance of G-to-T mutations in cell and animal model systems of AFB1 exposure (27, 28). Both reviews of human studies and mutation databases and a formal meta-analysis have confirmed the strong correlation of the 249^{Ser} TP53 mutation with aflatoxin exposure (3, 9, 29).

Our novel approach, quantitative SOMA, combines PCR coamplification of a known amount of internal standard plasmid and DNA extracted from plasma, with the mass spectrometric quantification of the relative amounts of WT, mutated, and internal standard DNA. The method is specific and, as applied here, was sensitive down to 500 copies of the mutated DNA per milliliter of plasma. In this study of 89 hepatocellular carcinoma cases, 42 cirrhotic patients and 131 nonliver diseased control subjects from a region of Africa with a high exposure to aflatoxin, we have shown a strong association of the plasma concentration of 249^{Ser}-mutated DNA with the progressive risk of hepatocellular carcinoma. Because of the relatively small numbers, particularly after adjustment, these risk estimates yielded results with wide confidence intervals. Nonetheless, we have clearly shown a consistent and reproducible association with hepatocellular carcinoma as well as a correspondence between increasing levels of this mutation in plasma and cancer outcome. In addition to an independent effect on hepatocellular carcinoma risk, we previously observed that the plasma 249^{Ser} mutation results in a multiplicative effect when exposure occurs in combination with chronic HBV infection (7).

The quantitative SOMA method presented here has significant potential for use in studies of early detection and selection of appropriate high-risk individuals for targeted intervention for hepatocellular carcinoma. In general, hepatocellular carcinoma presents at very advanced stages with little opportunity for curative treatment and extremely poor survival (23, 30). However, in the setting of small hepatocellular carcinoma (generally <2 cm in diameter), opportunities for surgical or percutaneous therapies show some therapeutic promise and potential survival benefit (31). The data currently available on the utility of novel markers for the early detection of hepatocellular carcinoma are limited by a lack of specificity and sensitivity as well as by the relatively small number of subjects in each study (32). The situation with TP53 249^{Ser} mutation in plasma DNA may be more promising, particularly in regions like Africa and Southeast Asia, because there is substantial evidence that this genetic alteration is relevant to the development of hepatocellular carcinoma and it reflects the cumulative exposure to aflatoxin B_1 (33). Incorporation of plasma TP53 249^{Ser} levels in combination with other potential hepatocellular carcinoma biomarkers into an algorithm might improve on the poor predictive value of α-fetoprotein determinations alone. Further longitudinal investigation of changes in the slope of plasma levels of TP53 249^{Ser} DNA over time may serve as a marker of malignant transition from DNA damage associated with chronic aflatoxin exposure or cirrhosis toward hepatocellular carcinoma. In addition, this marker may provide an objective metric for the early identification of high-risk individuals for targeted interventions. Further, using quantitative SOMA, the efficacy of interventions might be developed to use reduction of TP53 249^{Ser} levels as an intermediate end point in chemoprevention trials.

Finally, our results indicate that low levels (>500 copies/mL) of mutated TP53 249^{ser} DNA are detectable in a large proportion of nonliver diseased control subjects with dietary exposure to aflatoxin. Sixty-seven percent of hepatocellular carcinoma cases, 52% of cirrhotic patients, and 54% of control subjects had measurable plasma levels of TP53 249^{Ser} DNA. Only ~40% of tumors from hepatocellular carcinoma cases in The Gambia have been shown to contain the TP53 249^{Ser} mutation (7, 20). Interestingly, quantitative SOMA failed to detect TP53 249^{Ser} DNA in the plasma of nonliver cancer subjects from France, an area of extremely low exposure to aflatoxin (data not shown). Thus, irrespective of the predictive nature of this marker for hepatocellular carcinoma, the method may also have great potential for measuring lifetime, cumulative exposure to carcinogens, such as aflatoxin B_1, which produce characteristic point mutations in DNA in individual cells. Techniques to reduce or eliminate WT DNA, before PCR amplification through restriction digestion, are available and could permit the quantitative SOMA method to measure the low levels of mutated cells in a high background of WT cells (22, 34, 35). In addition, ongoing studies of chemoprevention to reduce aflatoxin exposure could benefit from using these markers as intermediate end points or in prioritizing individuals and communities most likely to benefit from preventive interventions (36).

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